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Acridones: A chemically new group of protonophores

(photosynthesis/electron transport/ionophores/uncouplers)

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ABSTRACT **Although the interaction of proton-conducting ionophores (protonophores) with photosynthetic electron transport has been extensively studied during the past decade, the mode of action of protonophores remained uncertain. For a better understanding of the molecular mechanism of the action of protonophores, the introduction of chemically new types of molecules will be required. In this work, we demonstrate that acridones (9-azaanthracene-10-ones) completely fulfill this requirement. At low concentrations of acridones, the thermoluminescence bands at +20°C and +10°C were strongly inhibited, while normal electron transport activity was retained. This indicates that the concentrations of S_2 and S_3 states involved in the generation of these bands are reduced. At higher concentrations, an increased activity of electron transport was observed, which is attributed to the typical uncoupler effect of protonophores. Indeed, acridones accelerate the decay of the electrochromic absorbance change at 515 nm and also inhibit the generation of the transmembrane proton gradient, measured as an absorbance transient of neutral red. Variable fluorescence induction was quenched even at low concentrations of acridones but was restored by either a long-term illumination or high light intensity. Acridones, similarly to other protonophores, promoted the autooxidation of the high-potential form of cytochrome b_{559} and partially converted it to lower potential forms. These results suggest that acridones, acting as typical protonophores, uncouple electron transport, accelerate the deactivation of the S_2 and S_3 states on the donor side, and facilitate the oxidation of cytochrome b_{559} on the acceptor side of photosystem II.**

The photosynthetic apparatus converts light into chemical energy by a series of reactions that gives rise to a coupled flow of electrons and protons, resulting respectively in the accumulation of reducing power (NADPH) and energy (ATP) (1). ATP formation requires an electrochemical proton gradient built up across the photosynthetic membrane that is generated by protons released from two sources: the photooxidation of water and oxidation of plastoquinol (PQH_2). PQH_2 can be oxidized via either the cytochrome b_6/f complex (2) or the oxidation–reduction of cytochrome b_{559}, which is part of a cyclic electron flow around photosystem II (PSII) (3). It was previously demonstrated that proton-conductive uncouplers (protonophores) facilitate the oxidation of PQH_2 and the autooxidation of cytochrome b_{559} (4, 5), but the action mechanism of this reaction remained unclear.

The known protonophores: ANT-2p [2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene]; CCCP [carbonylcyanide *m*-chlorophenylhydrazone]; FCCP [carbonylcyanide *p*-trifluoromethoxyphenylhydrazone]; SF 6847 [2,6-di-*t*-butyl-4-(2′,2′-dicyanovinyl)phenol] are polysubstituted phenol derivatives and chemically very similar. For a better understanding of the molecular mechanism of action of protonophores, the introduction of chemically new types of molecules is required.

In this study we present evidence that acridones (9-azaanthracene-10-ones) (6) operate as typical protonophores, accelerating the decay of the electrochromic absorbance transient and inhibiting the buildup of the transmembrane ΔpH. It is also shown that these agents uncouple photophosphorylation and act as an ADRY reagent (accelerators of deactivation reactions of the water-splitting enzyme, Y). Acridones also facilitate the oxidation of the high potential form of cytochrome b_{559} and its conversion into intermediary- or low-potential forms.

MATERIALS AND METHODS

Peas (*Pisum sativum* cv. Rajnai törpe) were grown under standard greenhouse conditions, and leaves were freshly harvested before each experiment. Intact chloroplasts were isolated essentially as described by Thorne *et al.* (7), and the chlorophyll content of the samples was estimated as described by Arnon (8).

The rate of photosynthetic oxygen evolution and uptake was measured as described by Droppa *et al.* (9) by using a Clark-type O_2 electrode (Rank Brothers, Cambridge, U.K.). Different parts of the electron transport chain were studied by the addition of various electron donors and acceptors: 2 mM NaN_3 and either 2 mM $K_3[Fe(CN)_6]$ or 0.1 mM methyl viologen were used to assay the whole electron-transport chain. Photosystem I (PSI) or PSII electron transport was measured by using 0.25 mM *p*-benzoquinone or 2,5-dichloro-*p*-benzoquinone, 40 μM dichlorophenolindophenol, and 2 mM ascorbate, depending on which system was being studied. In specific instances, PSII activity was also measured spectrophotometrically following the photoreduction of dichlorophenolindophenol at 590 nm in the presence and absence of 0.5 mM *sym*-diphenylcarbazide.

Thermoluminescence was measured in the temperature interval from −80 to +80°C with an apparatus similar to that described by Vass *et al.* (10). Samples were illuminated with white light of 10 $W \cdot m^{-2}$ for 2 min during continuous cooling from +20 to −80°C and then heated at a constant rate of 20°C/min to measure glow curves. Chemicals were added before the illumination started (11).

Fluorescence induction was measured by using a chlorophyll fluorimeter (Biotechnika RT, Szeged, Hungary). Samples containing chloroplasts corresponding to 10 μg of chlorophyll per ml were excited with red light (660 ± 10 nm) after 3 min of dark adaptation.

Abbreviations: ADRY, accelerators of deactivation reactions of the water-splitting enzyme Y; ANT-2p, 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; F_o, nonvariable fluorescence; F_{var}, variable fluorescence; PQH_2, plastoquinol; PSI and PSII, photosystems I and II; Q_A and Q_B, the primary and secondary quinone acceptor of PSII; SF 6847, 2,6-di-*t*-butyl-4-(2′,2′-dicyanovinyl)phenol; Acr-155, 4-bromo-2,5,7-trinitroacridone.

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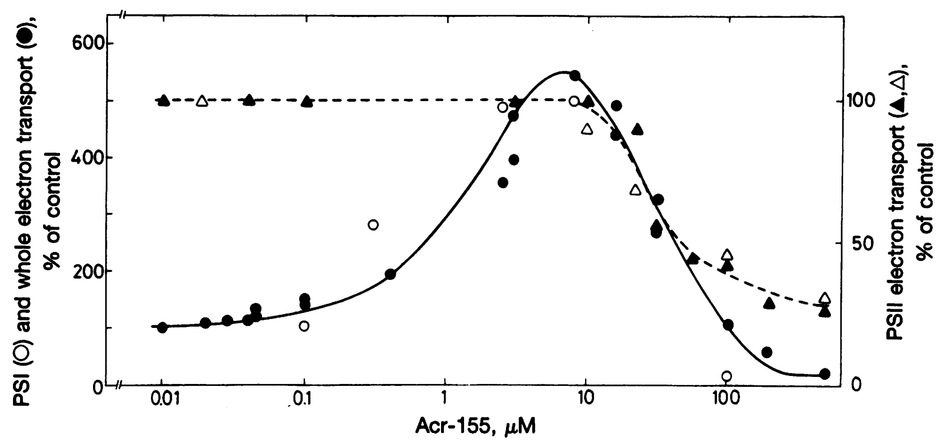

FIG. 1. Effect of 4-bromo-2,5,7-trinitroacridone (Acr-155) on the photosynthetic electron-transport activity of isolated chloroplasts. The reaction mixture contained osmotically disrupted chloroplasts (equivalent to 17 μg of chlorophyll per ml), 0.1 M sorbitol, 4 mM $MgCl_2$, 20 mM NaCl, 10 mM K_2HPO_4, 2 mM EDTA, and 50 mM Hepes (pH 7.5). Whole electron transport (●) was measured in the presence of 2 mM $K_3[Fe(CN)_6]$, PSI electron transport (○) was assayed by adding 0.1 mM methyl viologen and 2 mM NaN_3 together with 40 μM dichlorophenolindophenol and 2 mM ascorbate. PSII activity was measured in the presence of 0.25 mM *p*-benzoquinone (▲) or 2,5-dichloro-*p*-benzoquinone (△).

The amounts of the high- and low-potential forms of cytochrome b_{559} were determined spectrophotometrically in a Shimadzu UV-3000 spectrophotometer by following a procedure described earlier (12).

Proton release inside the thylakoids was detected by collecting the flash-induced absorbance changes of neutral red at 553 nm. The reaction medium contained 0.35 M sorbitol, 1 mM Hepes (pH 7.4), and 20 mM neutral red. The chlorophyll concentration of the sample was adjusted to 30 μM (13).

Flash-induced absorbance transients at 515 nm, due to electrochromic absorbance shift, were recorded as described earlier (14). The frequency of the exciting flashes was 1 s^{-1}. Generally, 30–50 kinetic traces were averaged in the multichannel signal averager.

RESULTS AND DISCUSSION

Acridones were previously found to inhibit PSII electron transport, but their effect on the whole electron transport activity has not been investigated (15).

The effect of 4-bromo-2,5,7-trinitroacridone (Acr-155) on the various parts of photosynthetic electron transport is shown in Fig. 1. Both overall and PSI-specific electron-transport activities were stimulated by increasing the acridone concentration up to 8 μM. Similar enhancement in PSII electron transport was not observed. At acridone concentrations greater than 8 μM, however, electron transport activity was strongly inhibited (15).

The above described increase of electron transport is characteristic for various uncouplers (16). The effect of acridones on the electron-transport activity was similar to that of the lipophilic uncouplers CCCP and FCCP (16) (Fig. 2). In accordance with this observation, we found that acridones abolished the proton gradient across the thylakoid membrane, as indicated by the drastic decrease of the flash-induced absorbance change of neutral red (Fig. 3). This ability of acridone was also confirmed by measuring the absorbance change at 515 nm. The amplitude of the absorbance change was strongly reduced by accelerating predominantly the decay of the signal upon addition of acridone (Fig. 3). These effects of ΔpH and $\Delta\Psi$ were similar to those obtained with CCCP, FCCP, and ANT-2p (data not shown).

The inhibited electron-transport activity, measured from H_2O to dichlorophenolindophenol, cannot be restored by addition of diphenylcarbazide, which donates an electron to

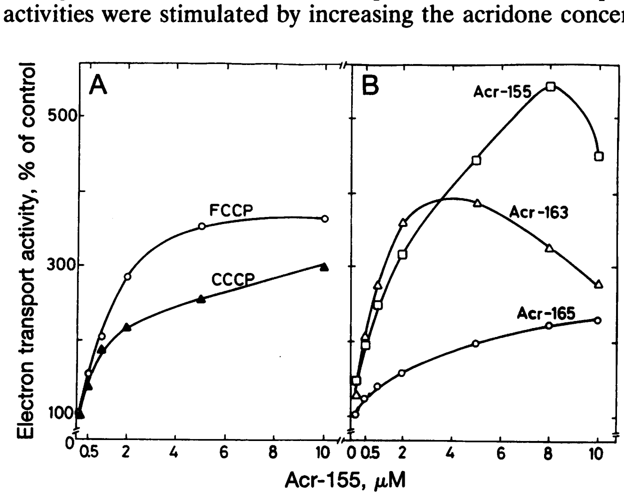

FIG. 2. Influence of lipophilic uncouplers (CCCP, FCCP) and different acridone derivates (Acr-155; Acr-163, 2,4,5,7-tetranitroacridone; and Acr-164, 2-bromo-4,5,7-trinitroacridone) on the photosynthetic electron transport activity measured from H_2O to $K_3[Fe(CN)_6]$. Experimental conditions were as in Fig. 1.

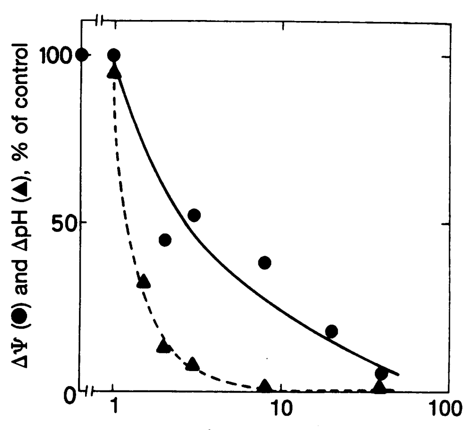

FIG. 3. Effect of Acr-155 on the transmembrane proton gradient (ΔpH) and electric field ($\Delta\Psi$) as measured by flash-induced absorbance changes of neutral red at 553 nm and carotenoid bandshift at 515 nm, respectively. The assay medium for ΔpH measurements contained 0.35 M sorbitol, 1 mM Hepes (pH 7.4), 20 μM neutral red, and chloroplasts equivalent to the chlorophyll concentration of 30 μM. The 515-nm absorbance change was measured in the medium described in Fig. 1. The frequency of excitation was 1 s^{-1}, and 30–50 kinetic traces were averaged for each point.

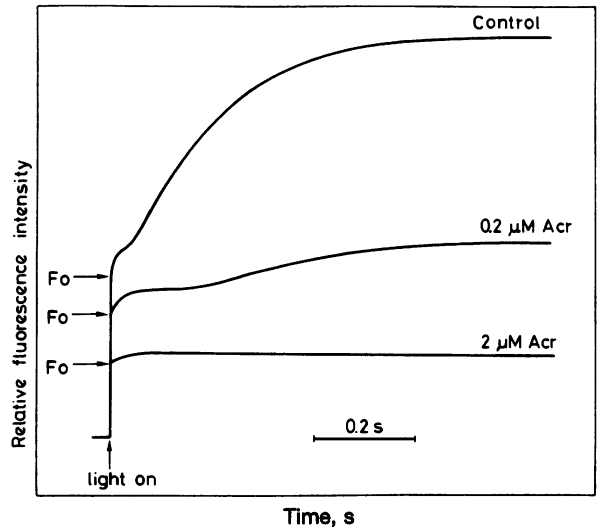

FIG. 4. Quenching of the fluorescence induction by acridone-155 in the absence of electron acceptors. Samples containing chloroplasts (equivalent to 10 μg chlorophyll per ml) were illuminated with 660 ± 10 nm red light. Intensity of excitation was 50 microeinsteins (μE)·m^{-2}·s^{-1}; 1 E = 1 mol of photons. Other experimental conditions were as in Fig. 1.

the *P*680 reaction center chlorophyll (17). This result is in accordance with the previous observation (18) that the uncoupler, ANT-2p, inhibited the diphenylcarbazide-supported dichlorophenolindophenol reaction, indicating interaction of acridone with the reaction center.

It has been demonstrated (4) that protonophores quenched the variable part of fluorescence (F_{var}) induction. Fig. 4 shows that in the absence of an electron acceptor, acridone is similar to FCCP and SF 6847 in dramatically quenching the yield of F_{var}, signifying that accumulation of the primary quinone acceptor of PSII, Q_A^-, was abolished (4). Addition of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) to the acridone-treated sample did not restore the F_{var} (data not shown) as observed with FCCP and SF 6847 (4). This result indicates that acridones might oxidize not only PQH_2 but also Q_A^- as well. Either long-term illumination or high light intensity could partly restore F_{var} resembling the effect of FCCP (Fig. 5). These results are in agreement with the fact that the inhibitory effect of protonophores is dependent on light intensity (4, 17). The fact that at >10 μM acridone concentrations, neither high light intensities nor long-term illumination could restore F_{var} suggests that acridone might block the Q_A^- formation itself.

Since FCCP, CCCP, and ANT-2p are widely used as proton-conducting ionophores to uncouple electron transport from ATP formation and are applied as ADRY reagents (19), we tested whether acridones as well possessed such a behavior. Thermoluminescence, which measures light emission originat-

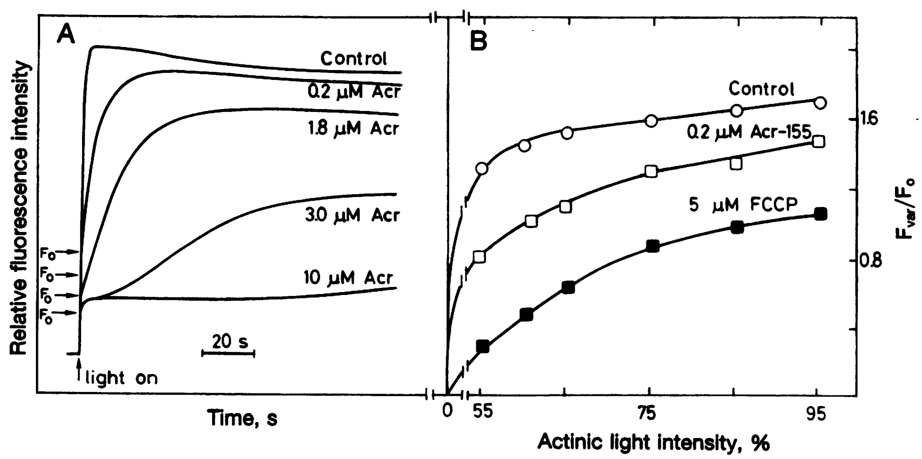

FIG. 5. Effects of the illumination time (*A*) and light intensity (*B*) on the rise of F_{var} in the presence of Acr-155 and FCCP. Maximal intensity of the actinic light was 148 μE·m^{-2}·s^{-1}. Other conditions were as in Fig. 4. F_o, nonvariable fluorescence.

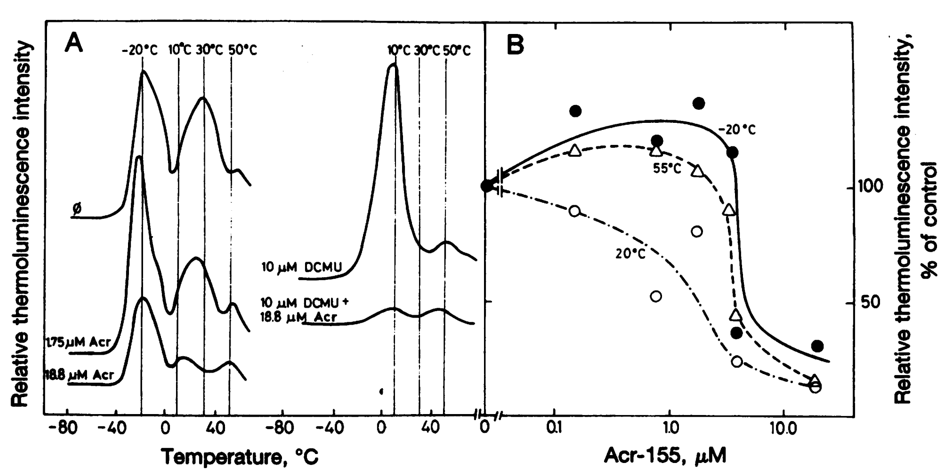

FIG. 6. Effect of Acr-155 on the thermoluminescence characteristics of isolated chloroplasts. The samples were cooled down from +20°C to −80°C during continuous illumination and heated up at the rate of 20°C per min in the dark. The chlorophyll concentration of the samples was 166 μg per ml. Other experimental conditions were as in Fig. 1.

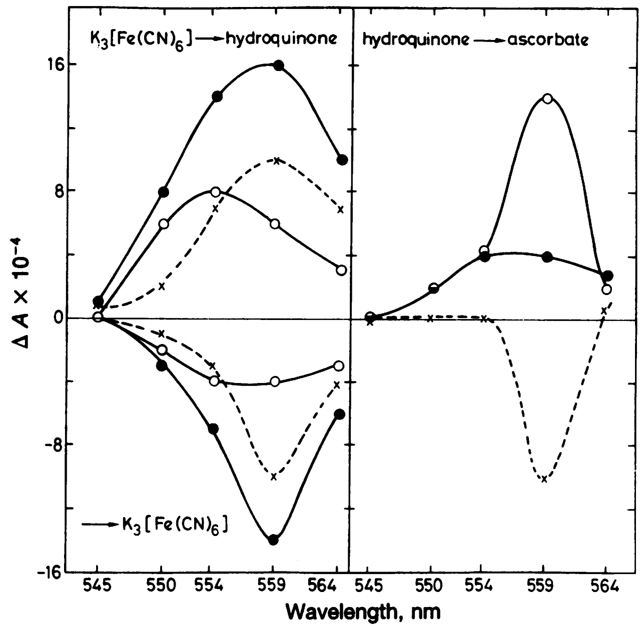

FIG. 7. Effect of Acr-155 on the chemically induced absorbance difference of cytochrome b_{559}. ●, Control chloroplasts; ○, chloroplasts treated with 20 μM Acr-155; ×, differences between ● and ○. Data points were obtained from transient absorbance after 10 ms of flash excitation. Chlorophyll concentration of the samples was 30 μM. The chemical treatments were made by adding 0.4 mM $K_3[Fe(CN)_6]$, 1.5 mM hydroquinone, and 5 mM sodium ascorbate. Other conditions were as in Fig. 1.

ing from recombination of positive and negative charges stored on the donor and acceptor sides of PSII (20), was used. As shown in Fig. 6, relatively low concentrations of acridone abolished both the B and Q bands of the glow curves, which correspond to $S_2S_3Q_B^-$ and $S_2(S_3)Q_A^-$ recombination, respectively (20). This is in agreement with the earlier observation that the powerful ADRY reagents ANT-2p or CCCP abolished both the B and Q bands by accelerating the reduction of oxidizing redox equivalents stored in S_2 and S_3 states within the water-splitting enzyme (21–23). In the concentration range affecting the Q and B bands, acridone even enhanced the intensity of the bands at −20°C and +50°C (Fig. 6). This effect can be explained, since ADRY agents generally accelerate the decay of higher S_2 and S_3 states by accumulating lower S_1 and S_0 states of the water-splitting enzyme (22, 24, 25). The bands at −20°C and +50°C originate from the charge recombination of tyrosine(Y_Z^+) or histidine(His^+)Q_A^- and tyrosine (Y_D^+)Q_A^-, respectively, as lower S states are enhanced (26, 27). Thermoluminescence data, together with the results of fluorescence induction and electron transport measurements, indicate that acridones act specifically as proton-conductive ionophores.

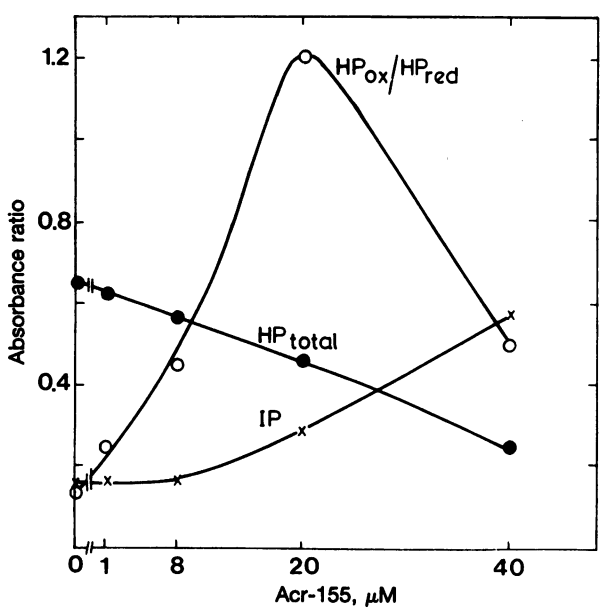

FIG. 8. The autooxidation of the high-potential form (HP) of cytochrome b_{559} and its conversion to the intermediary-potential form (IP) after treatment of the samples with Acr-155. Experimental conditions were as in Fig. 7.

Protonophores promote autooxidation of the high-potential form of cytochrome b_{559} that results in a partial conversion of the high-potential form to intermediary- and low-potential forms (12, 28). Therefore, chemically induced absorbance changes associated with cytochrome b_{559} were assayed to determine the amounts of different redox forms of cytochrome b_{559} induced by acridones. Fig. 7 shows that acridone oxidized about 70% of cytochrome b_{559} in the dark, and just a small proportion of the oxidized cytochrome b_{559} could be reduced by addition of hydroquinone. The extent of autooxidation of cytochrome b_{559} in the dark induced by acridone was similar to those observed with ANT-2p (12) or FCCP, CCCP, and SF 6847 (28). However, ascorbate completely reduced all oxidized cytochrome b_{559} (Fig. 8). This result indicates that acridone is similar to other protonophores in also inducing a partial conversion of the high-potential form to ascorbate-reducible intermediary- and low-potential forms (12, 28).

The aim of these investigations was to test the effects of acridones, the amino analogues of anthraquinone (15), on photosynthetic electron and proton-transport activity in isolated chloroplasts.

From our results, we have concluded that acridones act as protonophores by uncoupling electron and proton transport in thylakoid membranes. Like other protonophores, acridones (*i*) operate as ADRY reagents, deactivating the higher S states of the water-splitting enzyme, and (*ii*) also interact with the acceptor side of PSII and induce oxidation of PQH_2 via the oxidation of cytochrome b_{559}. It is likely that acridones with structures chemically different from known protonophores serve as tools to clarify the molecular mechanisms of protonophore action.

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1. Arnon, D. I. (1984) *Trends Biochem. Sci.* **9**, 258–262.
2. Lam, E. & Malkin, R. (1982) *FEBS Lett.* **144**, 190–194.
3. Arnon, D. I., Tsujimoto, H. Y. & Tang, G. M.-S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2942–2946.
4. McCauley, S. W., Melis, A., Tang, G. M. S. & Arnon, D. I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8424–8428.
5. Arnon, D. I. & Tang, G. M.-S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9524–9528.
6. Oettmeier, W., Masson, K. & Soll, M. (1992) *Biochim. Biophys. Acta* **1099**, 262–266.
7. Thorne, S. W., Horváth, G., Kahn, A. & Boardman, N. K. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3858–3862.
8. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15.
9. Droppa, M., Terry, N. & Horváth, G. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2369–2373.
10. Vass, I., Horváth, G., Herczeg, T. & Demeter, S. (1981) *Biochim. Biophys. Acta* **634**, 140–152.
11. Droppa, M., Horváth, G., Vass, I. & Demeter, S. (1981) *Biochim. Biophys. Acta* **638**, 210–216.
12. Barabás, K. & Garab, G. (1989) *FEBS Lett.* **248**, 62–66.
13. Garab, G., Farineau, J. & Hervo, G. (1987) *Photosynth. Res.* **11**, 15–27.

14. Barabás, K., Zimányi, L. & Garab, G. (1985) *J. Bioerg. Biomembr.* **17,** 349–364.
15. Oettmeier, W., Masson, K., Kloos, R. & Godde, D. (1992) in *Research in Photosynthesis*, ed. Murata, N. (Kluwer, The Netherlands), Vol. 3, pp. 563–566.
16. Arnon, D. I. & Tang, G. M.-S. (1985) *Biochim. Biophys. Acta* **809,** 167–172.
17. Packham, N. K. & Barber, J. (1984) *Biochem. J.* **221,** 513–520.
18. Packham, N. K. & Ford, R. C. (1986) *Biochim. Biophys. Acta* **852,** 183–190.
19. Renger, G. (1972) *Biochim. Biophys. Acta* **256,** 438–439.
20. Horváth, G. (1986) *Crit. Rev. Plant Sci.* **4,** 293–310.
21. Renger, G. & Eckert, H. J. (1981) *Biochim. Biophys. Acta* **638,** 161–171.
22. Renger, G. & Inoue, Y. (1984) *Biochim. Biophys. Acta* **725,** 146–154.
23. Demeter, S., Herczeg, T., Droppa, M. & Horváth, G. (1979) *FEBS Lett.* **100,** 321–324.
24. Renger, G., Bouges-Bocquct, B. & Delosme, R. (1973) *Biochim. Biophys. Acta* **292,** 796–807.
25. Hanssum, B., Dohnt, G. & Renger, G. (1985) *Biochim. Biophys. Acta* **806,** 210–220.
26. Debus, R. J. (1992) *Biochim. Biophys. Acta* **1102,** 269–352.
27. Demeter, S., Goussias, Ch., Bernát, G., Kovács, L. & Petrouleas, V. (1993) *FEBS Lett.* **336,** 352–356.
28. Barabás, K., Kravcova, T. & Garab, G. (1993) *Photosynth. Res.* **36,** 59–64.